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RESEARCH ARTICLE

Social organization and the evolution of life-history traits in two queen morphs of the ant *Temnothorax rugatulus*

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ABSTRACT

During the evolution of social insects, not only did life-history traits diverge, with queens becoming highly fecund and long lived compared with their sterile workers, but also individual traits lost their importance compared with colony-level traits. In solitary animals, fecundity is largely influenced by female size, whereas in eusocial insects, colony size and queen number can affect the egg-laying rate. Here, we focused on the ant *Temnothorax rugatulus*, which exhibits two queen morphs varying in size and reproductive strategy, correlating with their colony's social organization. We experimentally tested the influence of social structure, colony and body size on queen fecundity and investigated links between body size, metabolic rate and survival under paraquat-induced oxidative stress. To gain insight into the molecular physiology underlying the alternative reproductive strategies, we analysed fat body transcriptomes. Per-queen egg production was lower in polygynous colonies when fecundity was limited by worker care. Colony size was a determinant of fecundity rather than body size or queen number, highlighting the super-organismal properties of these societies. The smaller microgynes were more frequently fed by workers and exhibited an increase in metabolic activity, yet they were similarly resistant to oxidative stress. Small queens differentially expressed metabolic genes in the fat body, indicating that shifts in molecular physiology and resource availability allow microgyne queens to compensate for their small size with a more active metabolism without paying increased mortality costs. We provide novel insights into how life-history traits and their associations were modified during social evolution and adapted to queen reproductive strategies.

KEY WORDS: Metabolism, Life-history evolution, Trade-off, Longevity, Fecundity, Social insects, Gene expression

INTRODUCTION

In social insects, many life-history traits shifted and diversified during the evolution of their highly social lifestyle. The reproductive individuals, the queens, evolved to exhibit higher fecundity and extraordinarily long lifespans (Keller and Genoud, 1997), whereas their worker daughters often became sterile and short lived. Moreover, reproductive strategies can vary within a single caste in some social insects. For example, several ant species exhibit two different queen morphs, which vary in body size, mating and colony-founding behaviours. Body size is often negatively associated with fecundity in interspecific comparisons, whereas within the same species larger individuals with more body reserves have a higher reproductive success than smaller ones (Honěk, 1993; reviewed in Stearns, 1992). Differences in body size can also be a predictor of longevity, as larger individuals generally have a higher life expectancy compared with their smaller conspecifics (Calabi and Porter, 1989; Miller et al., 2002; Salaris et al., 2012; Urfer et al., 2011; reviewed in Samaras et al., 2002). This association is often attributed to the fact that larger organisms have a lower metabolic rate, which, according to the rate of living theory, confers a lower rate of reactive oxygen species production and a decelerated rate of ageing (Rubner, 1908; Loeb and Northrop, 1917; Pearl, 1922; Brys et al., 2007; reviewed in Speakman, 2005).

In social insects, selection is acting not only on the individual but also on the colony as a whole (Kramer et al., 2016; Negroni et al., 2016). The highly uneven distribution of reproduction among group members (reproductive skew; Keller and Reeve, 1994) and altruistic care of workers in insect societies result in a divergence in body size, fertility and lifespan between female castes. The divergence in life-history traits between queens and workers increases with increasing colony size across species. Moreover, colony-level traits such as colony size or queen number might even evolve to become more important individual traits like body size or age in these 'super-organismal' social insects (Korb, 2016). Investigating the influence of social organization on life-history traits in social insects can shed light on how proximate mechanisms including the physiological and molecular regulation of fecundity and longevity were reshaped during social evolution from a solitary ancestor (Rodrigues and Flatt, 2016). It is interesting to study the evolution of life-history trait divergence not only between the queen and worker castes but also within a single caste. In ants, queen number varies both between species and within a species. Indeed, the influence of social organization on queen fecundity has been intensely studied. These investigations revealed that queens in monogynous societies are able to lay a higher per-capita number of eggs compared with queens in polygynous societies. In many ant species, queen fecundity decreases with queen number per colony (*Paratrichina fulva*: Arcila et al., 2002; *Linepithema humile*: Keller, 1988; *Solenopsis invicta*: Vargo and Fletcher, 1989; Vargo, 1992; *Petalomyrmex phylax*: Dalecky et al., 2005; and *Cardiocondyla obscurior*: Schrempf et al., 2011). This decrease in fecundity might be in part caused by a reduction in worker care, but queen number also affects queen morphology and founding strategy (McInnes and Tschinkel, 1995; DeHeer and Tscbinkel, 1998; Howard, 2006; Sundström, 1995; reviewed in Heinze and Keller, 2000). Larger, well-provisioned queens are more likely to found new colonies independently and to reside as the sole reproductive individual in an ant colony. In contrast, smaller queen size is associated with multi-queen societies, which arise from the adoption of daughter queens. Queens from monogynous societies are under stronger selection to live long, as they are the only reproductive

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individuals (Keller and Genoud, 1997; Keller and Passera, 1989; Nonacs, 1988; Schrempf et al., 2011). Their demise eventually causes the death of the colony, resulting in the evolution of extraordinarily long lifespans of monogynous ant queens. Thus, species showing this social polymorphism are ideal for testing theories on the interaction between life-history traits.

Our model system, the facultative polygynous ant *Temnothorax rugatulus*, exhibits a bimodal distribution of body size in queens: large macrogynes and the smaller, worker-sized microgynes (Rüppell et al., 1998; Fig. 1A,B). As in most other ants with queen dimorphism, queen morph is associated with social organization and reproductive strategy (Rüppell et al., 2001a). The smaller *T. rugatulus* microgynes occur predominantly in polygynous societies and establish new nests by budding accompanied by part of the workforce from their mother's nest. In contrast, the larger macrogynes found new colonies independently after the mating flight and occur most often in monogynous colonies. These alternative reproductive strategies should lead to divergent life-history traits. In macrogynes, selection for high fecundity and longevity should be more important than in microgynes, which commonly share reproduction with other queens and therefore can be replaced by them (Negroni et al., 2016). In *T. rugatulus*, the proximate basis of queen morph is not entirely resolved, but there appears to be some heritability (Rüppell et al., 2001a). In contrast to other well-known cases with polymorphic queens, such as *S. invicta* and *Formica selysi*, where queen morph is genetically controlled by a single non-recombining genomic element, a social chromosome (Wang et al., 2013; Purcell et al., 2014; Yan et al., 2020; Brelsford et al., 2020), such a pure genetic control is unlikely in *T. rugatulus*. In the field, mixed nests with queens of both morphs are occasionally found.

Here, we explored how queen morph and queen number influence queen fecundity in the ant *T. rugatulus*, by manipulating colony composition in a factorial design and recording egg-laying rates and worker care directed towards the queens. Moreover, to characterize traits associated with these two alternative life-history strategies, we studied differences in metabolic rate between the two queen morphs, their survival under paraquat-induced oxidative stress and their fat body gene expression. We expected a lower egg-laying rate, increased metabolic rate and decreased oxidative stress resistance in microgynous queens. Microgynous queens, which are smaller, possibly attract less attention from their workers and have adapted to a life where they share reproduction with other queens, which can replace them. These differences in physiology should be reflected in morph-specific transcriptomes, probably indicating a higher activity of metabolic genes, but a relative downregulation of oxidative stress resistance genes. Based on earlier studies on other ants (Vargo and Fletcher, 1989; Schrempf et al., 2011), we predicted reduced egg-laying rates in polygynous versus monogynous societies and questioned whether queen number affects the two queen morphs differently. Finally, we manipulated colony size as this strongly varies in this species and is associated with queen number (Rüppell et al., 2001b). We expected that queens with a larger workforce at their disposal would lay more eggs. In this social species, we were interested in whether individual traits such as body size become less influential for life-history characters such as fecundity than colony level-traits, including colony size and social structure.

MATERIALS AND METHODS

Field collection and categorization of queen morph

The ant *Temnothorax rugatulus* (Emery 1895) occurs throughout western USA and Mexico, and lives in rock crevices or under stones in higher elevation oak and pine forests (Rüppell et al., 1998). We collected 536 colonies in Arizona in August 2015 at 15 sites throughout the Chiricahua Mountains (Table S1a,b). After transport to the laboratory, the ant colonies were kept in plastered nest boxes containing an artificial nest site consisting of a Plexiglas perimeter (3 mm high) with an entry, sandwiched between two microscope slides (7.5×2.5×0.5 cm). The ants were fed with crickets and honey twice weekly, provided with water *ad libitum* and maintained in a climate chamber at 22°C, on a 12 h light:12 h dark cycle.

We followed Rüppell et al. (1998) and calculated a body size index (*I*) for each queen (1464 queens from 536 colonies; Fig. 1B) from head width (HW), thorax width (TW) and thorax length (TL), measured under a stereo microscope at 200× magnification, using

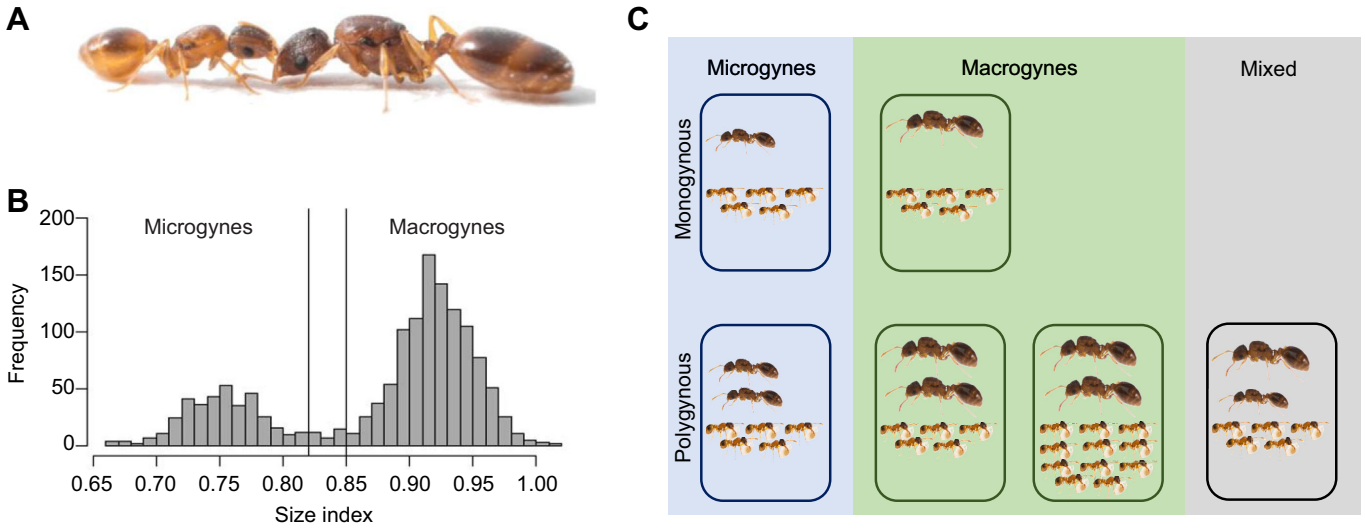

Fig. 1. Experimental design. (A) A microgyne (left) and a macrogyne (right) *Temnothorax rugatulus* queen. Photo: Romain Libbrecht. (B) Bimodal distribution of queen body size, as revealed by the body size index: $I=[\sqrt{(TL\times TW)}+HW]/2$. (C) Design of the queen fertility experiment. Blue, monogynous and polygynous treatments with microgyne queens; green, treatments with macrogyne queens (monogynous, polygynous and large colony); and grey, the mixed treatment with queens of both morphs. Colony size is indicated by the number of workers (five ants indicates 50 workers; 11 ants represents 125 workers). Photos: Barbara Feldmeyer.

the formula $I=[\sqrt{(TL\times TW)}+HW]/2$, a measurement that closely correlates with the queen's dry mass. For illustration of morphological differences, photos of a subsample (N=265 queens) are available from Google Drive (https://drive.google.com/drive/folders/1-lJVUCP0cbsM3knLPmKXjUEZmRdkfD4w?usp=sharing). The distribution of queen body size was bimodal with only a few queens falling in the centre of the distribution (Fig. 1B). Queens with an index <0.82 were grouped into the microgyne category, whereas queens with an index >0.85 were considered to be macrogynes. The colonies contained 75.4% macrogyne queens, 12.6% microgynes only, 8.8% were mixed colonies containing queens of both morphs, and 3.1% contained at least one queen with an undetermined morph lacking both macrogynes and microgynes (Table S1c). As expected, macrogynes resided more often in monogynous than polygynous colonies (62.3% versus 37.4%; χ^2-test, χ^2=22.7, d.f.=1, P<0.001), while the opposite pattern was found for microgynous colonies (36.7% monogynous versus 66.2% polygynous; χ^2-test, χ^2=7.2, d.f.=1, P<0.001).

Queen fecundity experiment

This experiment was designed to investigate the influence of body size, colony size, social structure and queen morph on behaviour and egg production (Fig. 1C). In addition to standardizing colony composition and maintenance, we used a full-factorial design to investigate the effect of social structure and queen morph and their interaction. We split 13 microgyne colonies (with 1–11 queens) and 12 macrogyne colonies (2–5 queens) into smaller experimental units, each receiving either one or two queens, 50 workers and 12 larvae, thereby creating polygynous and monogynous treatments for each queen morph. A mixed treatment was set up to study the influence of queen morph on fertility and reproductive skew; these units contained a macrogyne and a microgyne queen from 10 originally mixed colonies. Finally, to test for the effect of colony size, we added a treatment consisting of two macrogynous queens, 125 workers and 30 larvae derived from independent colonies (large colony treatment; Fig. 1C). Experimental colonies did not differ in their original worker or queen number between macrogynous, microgynous and mixed colonies (worker number: F=2.78, d.f.=2, P=0.08; queen number: χ^2=5.72, d.f.=2, P=0.06). Independent colonies were used for the large colony treatment and their original worker and queen number did not differ from those used in the polygynous and monogynous macrogyne treatments (worker number: F=0.02, d.f.=1, P=0.88; queen number: χ^2=0.57, d.f.=1, P=0.44). Each experimental colony received a similar proportion of workers from each worker caste (e.g. foragers, guards, brood-carers). We chose 50 workers as the colony size of our standard treatment, as *Temnothorax* colonies mature and start to produce sexuals in their 3rd to 4th year with a colony size of around 50 workers (Plateaux, 1986). The relatively low larvae to worker ratio was chosen in order to stimulate queen egg production (Negroni et al., 2021). We kept the ratio of workers to larvae the same in the treatments with varying worker numbers. As microgynes are rare in the field, we did not have sufficient microgyne colonies available to set up a large colony size treatment with microgynes.

In order to record egg production of individual queens in polygynous colonies, we used either of two lipophilic dyes (blue: Sudan Black, 33 g l^{-1}; red: Sudan IV, 40 g l^{-1}) 48 h before experimental colony establishment. Each queen was isolated from her colony and dye was administered by applying a mixture of sunflower oil and dye to the head of the queen. Queens ingested the dye by self-grooming, which resulted in a colour change of the cuticle (red or blackish), making them and all newly queen-laid eggs identifiable (either light red or blue; Fig. 2A,B; Stroeymeyt et al., 2007). We used two different colours for queens of polygynous colonies, and randomized queen colour over monogynous treatments and between morphs of mixed colonies (Table S1d).

Starting 24 h after experimental set-up, each coloured queen was individually observed for 5 min every other day, for 45 days (total observation time, 115 min per queen). For each 5 min scan, we recorded her walking time, the number and type of interactions and how often she was fed by workers (number of trophallaxis events). Additionally, we recorded the number of coloured eggs and identified their origin (i.e. red eggs were produced by a red queen). The distance between polygynous queens was measured under a stereomicroscope. To determine colony productivity, the entire brood of a colony was weighed on the first and last day of observation.

Statistical analyses were conducted in R v.3.3.2 (http://www.R-project.org/) using the packages car and lme4. In order to investigate the individual contribution of polygynous queens to colony egg production, we calculated the reproductive skew as skew=$N_b\times V_b$, where N_b is number of of queens and V_b is the variance in reproductive output among queens (Pamilo and Crozier, 1996). We analysed differences in skew between polygynous treatments (including the mixed treatments; Fig. 1C), with an LM using reproductive skew as response variable and treatment as an explanatory variable. We tested whether macrogynes have a higher egg-laying rate in mixed colonies compared with the smaller microgynes by performing a unilateral Student's test comparing the proportion of eggs produced by macrogynes against 0.5, after we verified data normality (Shapiro test: W=0.94, P=0.44). In a separate analysis, we investigated the influence of colony size on egg production and reproductive skew focusing on the macrogynous and polygynous treatments only. Thus, we compared macrogyne colonies from the polygynous treatment with macrogynes from the large colony treatment that was also polygynous (Fig. 1C). For this, we ran two models (LM) with colony size treatment as a response variable, one with the number of eggs produced per colony and the other with reproductive skew as a response variable. All other models were implemented with original colony identity (ID) as a random factor to account for the split colony design (linear mixed-model, LMM; generalized linear mixed-model, GLMM). For models differentiating individual queen traits, we added fragment ID (experimental colony ID) as a second random factor. To test for the combined effect of queen number and body size on colony egg production, we ran a GLMM with the number of eggs produced per colony as a response variable and social structure in interaction with queen morph as explanatory variables. On the individual level, we ran four additional GLMMs with (i) number of eggs produced, (ii) queen mobility (movement time ratio), (iii) queen grooming rate by workers and (iv) trophallaxis rate as response variables, and queen morph in interaction with social structure as explanatory variables. Before the analysis, feeding rate and mobility data were square root transformed. In case of over-dispersion, we used the penalized quasi-likelihood method (function glmmPQL) available in R package MASS. *Post hoc* pairwise comparisons were performed using the package multcomp with Bonferroni correction.

Metabolic rate and survival under oxidative stress

In this experiment, we compared mass-specific metabolic rate, egg production and survival under paraquat-induced oxidative stress between the two queen morphs. We used queens from 58 colonies (36 macrogynes, 15 microgynes, seven mixed colonies) and created a total of 60 macrogynous and 23 microgynous experimental units by colony splitting and/or queen, brood and worker removal, each

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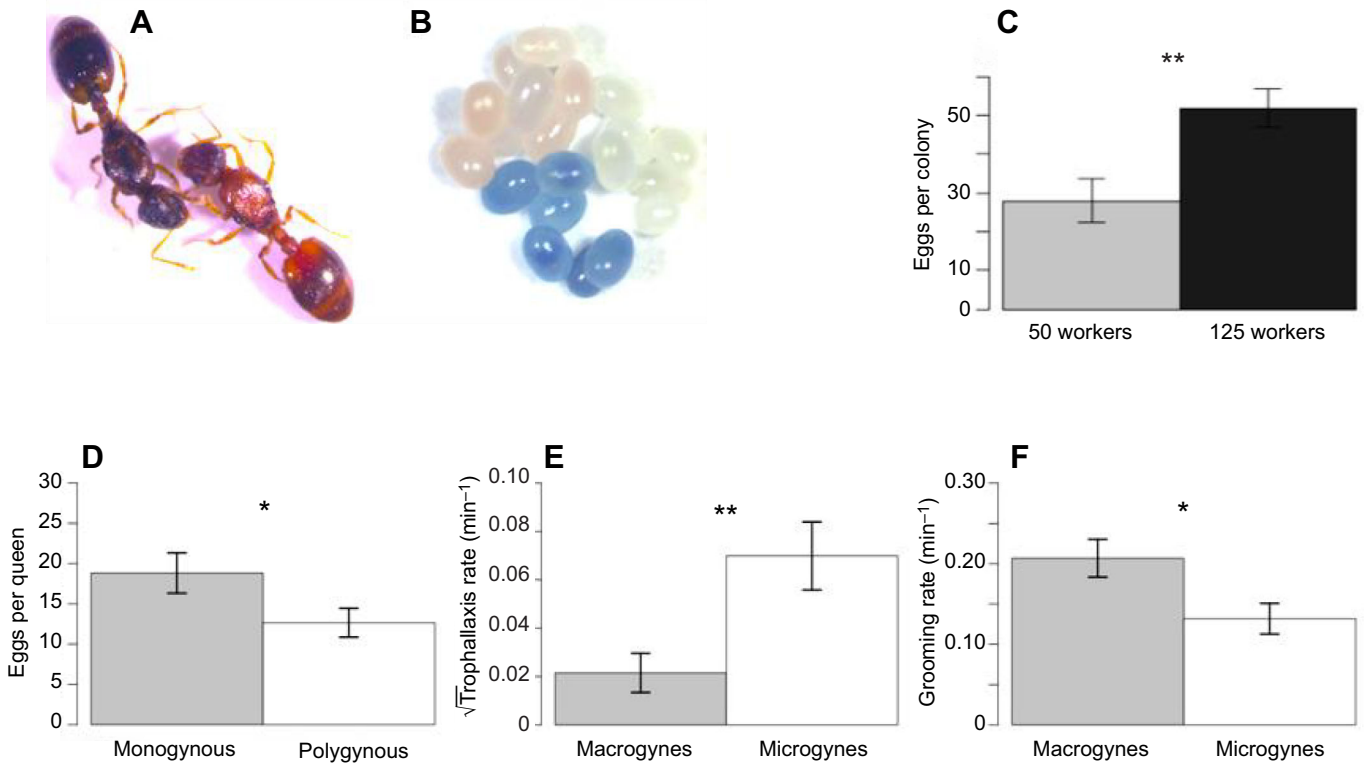

Fig. 2. Egg production of and care provided to individual queens in polygynous colonies. (A,B) Coloration with lipophilic dye of (A) queens (left, Sudan Black; right, Sudan IV) and (B) eggs (right, uncoloured; top left, Sudan IV; bottom, Sudan Black). (C) The number of eggs produced in a colony depends on worker number (LM: F=9.69, d.f.=1, P<0.01). (D) Queens lay more eggs when they reside alone in a colony. (E,F) Macrogyne queens are (E) fed less often, but (F) groomed more often than microgynes. Statistical results of the full model are presented in Table 1. *P<0.05, **P<0.01.

containing one queen, 14 workers and 10 larvae. A balanced composition of the workforce was ensured by allocating a similar number of workers of different castes to each treatment. The experimental units were kept at 25°C on a 12 h light:12 h dark cycle for 20 days.

Twenty days after creation of the experimental units, we measured oxygen consumption for each queen separately (45 macrogynes and 23 microgynes) using an oxygen microsensor (MicroRespiration system, UNISENSE, Aarhus, Denmark). Real-time O_2 concentration in a glass micro chamber (volume 0.448 ml, sealed with paraffin oil; lid hole previously filed with agar, 0.5%) containing the queen, and placed in a water bath at 22°C, was recorded for 10 min, using the oxygen microsensor and the software SensorTraceBasic v.3.0.200 (UNISENSE). All queens were weighed directly after metabolic rate measurement (PESCALE Wägetechnik, Bisingen, Germany; accuracy 1 µg). There was no overlap in mass between queen morphs (macrogynes: 1.680±0.148 mg; microgynes: 0.922±0.142 mg, means±s.e.m.). We calculated the mass-specific respiration rate by taking the slope of O_2 (O_2 concentration×chamber volume) over time, with a fixed time range of 9 min, corrected for the queen's body mass. This mass-specific respiration rate was used as a proxy for metabolic activity per mass unit, i.e. metabolic rate (Calabi and Porter, 1989).

In order to see whether the scaling between body mass and metabolic rate within *T. rugatulus* ant queens was similar to the scaling observed across insect species, we graphically compared our queen morph-specific metabolic rate measurements with those in Chown et al. (2007). To do this, we converted metabolic rate data to microwatts assuming joule equivalence of 20.7 kJ l^{-1} O_2. As temperature affects metabolic activity in insects, we selected metabolic measurements conducted at 20°C from Chown et al. (2007), being the closest available to the 22°C used for our own measurements.

As *Temnothorax* queens can live for up to two decades (Plateaux, 1986) and very few queens died over the year of colony maintenance, it was not feasible to investigate whether the two queen morphs that differed in body mass and metabolic rate (see below) also differed in lifespan. We therefore artificially induced oxidative stress using paraquat, which is a commonly used procedure (Langberg et al., 2018; Song et al., 2020), though we acknowledge that the effects of paraquat on study organisms can be diverse and our findings have to be interpreted with care (Meitern et al., 2013). The experimental colonies were divided into three treatments: (1) paraquat-microgyne treatment including all 23 microgynous colonies, (2) paraquat-macrogyne treatment including 29 macrogynous colonies, and (3) control without paraquat with 19 macrogynous colonies. Our paraquat treatments consisted of administering a single dose of paraquat (pure from Sigma-Aldrich) solution mixed with sunflower oil, every second day to each queen until the end of the experiment. The macrogyne control received oil only.

To administer the paraquat–oil solution, each queen was isolated in a Petri dish and the head of the queen was covered with a paraquat–oil solution (treatment) or oil only (control) by using a thin needle. The queens readily started grooming themselves and we ensured by direct observation that they ingested the solution before being placed back into the colony after 45–60 min. As the amount of oil applied relative to body mass was lower in macrogynes (LMM: χ^2=6.38, d.f.=1, P=0.01), we used a concentration of 0.8 mol l^{-1} paraquat solution for macrogynes and 0.46 mol l^{-1} paraquat for microgynes. The

concentration of 0.8 mol l^{-1} paraquat was established to have sublethal effects in a preliminary experiment with macrogyne queens and downscaled for microgynes to obtain a similar average concentration per body mass of 17 µmol g^{-1} (Table S2a). To measure queen fecundity, we counted the number of eggs in each experimental colony the day before the paraquat treatment and then every second day. We recorded queen survival every day from November 2017 to February 2018. The experiment ended on day 44, when all queens from the paraquat treatments had died.

We compared fecundity and metabolic rate between queen morphs (GLMM and LMM, respectively). We analysed the effect of treatment on queen survival (survival model with the R package coxme). To analyse the influence of paraquat treatment in interaction with queen morph on queen egg production, we created a categorical variable 'paraquat treatment', which included two levels (yes or no) differing in time: day zero (one day before starting the treatments: no) and day 10 (after 10 days at least 72% of each queen morph were still alive: yes). Day 10 as the second factor level was chosen to optimize statistical power, i.e. a compromise between the period of paraquat exposure and queen survival (at least 72% of each queen morph still alive). We analysed the influence of queen morph (paraquat-macrogyne/paraquat-microgyne) in interaction with paraquat treatment on queen egg production. We ran a LMM with egg number as the response variable and morph (paraquat-macrogyne/paraquat-microgyne) in interaction with paraquat treatment as the explanatory variable (Table S2b, Fig. S2d). Queen survival was not linked to metabolic rate (χ^2=2.48, d.f.=1, P=0.12) or to its interaction with the treatment (χ^2=1.90, d.f.=3, P=0.28).

Gene expression comparison between queen morphs

To investigate gene expression differences between queen morphs, we used 14 polygynous colonies, 8 with macrogynous queens and 6 with microgynous queens. We standardized the queen number to two, worker number to 50 and larvae number to 12. Experimental colonies were maintained at 22°C, on a 12 h light:12 h dark cycle for 7 weeks, then transferred to artificial winter conditions (5°C) for 12 weeks. After 16 additional weeks at 22°C, we randomly chose eight macrogynes and six microgynes, each queen from a different colony. The ants were killed by decapitation, and the fat body was dissected on ice and put in Trizol (Invitrogen). The RNA was extracted using the RNeasy mini kit (Qiagen), resulting in 14 RNA samples. Library construction and RNA-sequencing were conducted at BGI Hong Kong (100 bp paired reads, Illumina HiSeq 2000/2500; see Data availability section).

Sequences were trimmed with Trimmomatic (Bolger et al., 2014) and checked for quality using FastQC v.0.11.5 (https://github.com/s-andrews/FastQC). A transcriptome was *de novo* assembled using Trinity v.2.4.8 (Haas et al., 2013), resulting in an assembly with 166,120 contigs (quality statistics: Table S3d, back mapping rates: Table S3e). To eliminate spurious reads, all contigs with fewer than 10 reads in at least four samples were removed, as were contigs with a Cook's distance above 72, the point where the expression of highly variable genes increased (Alleman et al., 2019). These filtering steps reduced the count matrix to 67,000 contigs; 55.86% of the contigs were annotated with BlastX v2.9.0, only considering those hits with an E-value below 10^{-5} against the non-redundant insect database (Altschul et al., 1990). The read count estimates per contig and sample were obtained using RSEM v.1.3.0 with the implemented Bowtie2 aligner (Li and Dewey, 2011; Langmead and Salzberg, 2012). To remove putative contaminant sequences from other organisms, we decided to align the *de novo* transcriptome against a custom database containing different insect genomes using BlastN. Only transcripts which had at least one match with an E-value below 10^{-5} were kept for further analysis and the read count matrix was filtered accordingly. The principal component analysis (PCA) and the gene expression analysis were performed in R version 3.6.1 (http://www.R-project.org/) using the plotPCA and contrast function of Deseq2 v.1.24.0 (Love et al., 2014). Gene ontology (GO) annotation (Ashburner et al., 2000) was conducted using InterProScan v.5.34-73.0 (Jones et al., 2014) on the translated amino acid sequences using Transdecoder v.5.5.0 and GO enrichment analysis was performed using the R package topGO v.2.36.0 (https://bioconductor.org/packages/topGO/). We additionally obtained GO annotations and functional information from the UniProt database (www.uniprot.org), using *Drosophila melanogaster*, *Homo sapiens* and *Mus musculus* as model organisms (custom script, see Data availability section) for all annotated differentially expressed genes (FDR P<0.05).

As both the fire ant *S. invicta* and *T. rugatulus* are Myrmicine ants, we were interested whether among the differentially expressed genes between microgynes and macrogynes there were more genes than expected by random that have homologues located on the social chromosome of the fire ant, which determines queen morph in this species. We used the genome and the published list of genes on the inversions of the social chromosome (Yan et al., 2020) to check for overlap between these genes and the differentially expressed genes between macrogynes and microgynes. We performed a BlastN homology search using our *de novo* transcriptome as well as the genome of *S. invicta* together with the positions of genes located on the social chromosome inversions (Yan et al., 2020). We used non-default parameters for the E-value cut-off (10^{-5}) and percentage identity (95%). Afterwards, we created a BED file based on the BLAST output using the start and end position of the gene in the *S. invicta* genome as coordinates and looked for overlap with the genes on the inversions using bedtools v.2.29.2 (Quinlan and Hall, 2010). To test whether there were more differentially expressed genes on the social chromosome than expected, we performed Fisher's exact test in R.

RESULTS

Queen fecundity experiment

The number of eggs was not affected by social structure, queen morph or their interaction (LMM, structure: χ^2=2.49, d.f.=1, P=0.11; morph: χ^2=0.12, d.f.=1, P=0.72; interaction: χ^2=1.36, d.f.=1, P=0.24; Fig. S1a; Table 1). The egg-laying rate of individual queens was reduced in polygynous colonies, but was unaffected by morph (Table 1, Fig. 2D). The reproductive skew among nestmate queens was moderate with an average of 0.31; that is, 71% of all eggs were produced by the most reproductive queen. However, the skew differed from zero for all treatments (polygynous-macrogynes: t=5.76, P<0.001; polygynous-microgynes: t=3.52, P=0.001; mixed: t=6.36, P<0.001), but did not vary between polygynous treatments (LM: F=2.45, d.f.=2, P=0.10; Fig. S2b). Moreover, macrogynes did not lay more or fewer eggs than microgynes in mixed colonies (macrogynes: mean 0.57, 95% confidence interval CI=0.32%; Student's test: t=0.59, d.f.=10, P=0.28). The skew was unaffected by the number of workers in the nest per queen when comparing the polygynous-macrogynous treatments (LM: F=1.33, d.f.=1, P=0.26; Fig. S2b). Yet, macrogyne queens in larger colonies produced more eggs (LM: F=9.69, d.f.=1, P<0.01; Fig. 2C). The spatial distance between queens in polygynous colonies did not affect the reproductive skew (LM: χ^2=3.04, d.f.=1, P=0.09; Fig. S2a), but was negatively correlated with the number of eggs produced (LM: F=4.76, d.f.=1, P=0.04; Fig. S2b).

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Table 1. Influence of social structure, queen morph and their interaction on individual egg production, grooming, feeding rate and mobility in *Temnothorax rugatulus*

	Egg number			Grooming rate			Trophallaxis rate			Movement rate		
Fixed variables	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Morph	0.96	1	0.75	5.02	1	0.03	9.71	1	<0.01	0196	1	0.16
Structure	5.98	1	0.01	1.36	1	0.24	0.01	–	0.98	0.81	1	0.36
Morph×Structure	1.68	1	0.19	0.00	1	0.96	0.08	–	0.77	0.18	1	0.66

Significant *P*-values were corrected for multiple testing using the Bonferroni method.

Behavioural observations revealed that microgynous queens were fed more often by workers than macrogynes, independent of social structure (Table 1, Fig. 2E). Larger macrogynous queens were groomed more often than the smaller microgynes (Table 1, Fig. 2F). Grooming and feeding rates were unaffected by social structure or their interaction with queen morph (Table 1). No aggression between queens was observed in polygynous colonies. Colony size did not affect the rate at which queens were groomed or fed by workers (respectively: W=253.5, P=0.75; d.f.=1, χ^2=0.40, P=0.53). Finally, queen movement was independent of social structure, queen morph or their interaction (Table 1).

Metabolic rate and survival under oxidative stress

Macrogyne queens had about twice the body mass of microgynes (Fig. 3A), but the two morphs did not differ in egg production (GLMM with Poisson distribution: χ^2=0.22, d.f.=1, P=0.64; Fig. 3B). Microgynes had a metabolic rate twice as high as that of macrogynes (LMM: χ^2=58.30, d.f.=1, P<0.001; Fig. 3C), as was expected by the size relationship between the two queen morphs (Chown et al., 2007). Both queen morphs had a lower survival rate under paraquat-induced oxidative stress, but there was no difference in survival between morphs, with microgynes surviving marginally better than macrogynes (survival mixed-model: χ^2=33.11, d.f.=2, P<0.001; paraquat-macrogyne versus oil-control: Z=4.91, P<0.001; paraquat-macrogyne versus paraquat-microgyne: Z=−2.64, P=0.06; Fig. 3D). Queen survival was not linked to metabolic rate (χ^2=0.87, d.f.=1, P=0.35) nor was it linked to the interaction with treatment (χ^2=1.90, d.f.=2, P=0.39). Finally, egg production was influenced by an interaction between treatment and morph, with microgynes showing a decrease in the egg-laying rate after 10 days of daily paraquat administration (Z=3.09, P=0.007), whereas macrogynes maintained a constant egg-laying rate (Fig. 3E).

Gene expression analyses

A PCA on read counts of all transcripts revealed a strong clustering of samples according to queen morph (Fig. 4A). The two queen morphs differed in the expression of 587 genes, 262 being up-regulated in macrogynes and 325 up-regulated in microgynes (Table S3a,b). Among the top 10 differentially expressed genes according to log fold-change, we found GRB10-interactionGYF protein 2 to be up-regulated in macrogynes, which is involved in the insulin-signalling pathway according to the UniProt functional annotation (Table S3c). A gene annotated as *proteasome activator complex subunit 4B-like* was upregulated in macrogynes. The functional enrichment analysis revealed that genes involved in metabolic processes or the regulation of cellular processes were differentially expressed between queen morphs. Over-represented functions in upregulated genes of microgynes belonging to metabolism included *malate metabolic process* and *fatty acid metabolic process* (Fig. 4B; Table S3e). The enrichment of *proteasome assembly* in macrogynes revealed that these larger queens degrade more proteins (Fig. 4B; Table S3d). Moreover, the enrichment of *glycerol-3-phosphate catabolic process* in microgynes (Fig. 4B) indicated a higher energy consumption (Berg et al., 2002). In total, we found five queen-specific differentially expressed genes in *T. rugatulus*, which map to *S. invicta* genes located on the social chromosome. However, this was not more than expected given the number of genes in the fire ant genome (Fisher's exact test: odds ratio=0.19, P=1.0).

DISCUSSION

The evolution of life-history traits has been intensely studied in solitary insects. Less attention has been paid to social insects, which show a great diversity of phenotypes with divergent life-history strategies. Here, we focused on two queen morphs in the ant *T. rugatulus* that differ in body size, colony-founding behaviour and, consequently, the social organization of their societies. Our experimental study reveals that queen body size plays a minor role in comparison to social organization and colony size. Moreover, we reveal that physiological parameters such as metabolic rate and the expression of metabolic genes shifted during the evolution of these alternative reproductive morphs. Furthermore, we identified genes associated with the different queen morphs and their respective phenotypes, e.g. small queens with a higher metabolism also upregulate metabolism genes in comparison to expression in large queens.

Social organization affected the number of eggs laid per queen, with higher per capita egg numbers in monogynous colonies. This is in accordance with earlier work on other ant species, which revealed that queen fecundity decreases with the number of queens per colony (Arcila et al., 2002; Keller, 1988; Vargo and Fletcher, 1989; Vargo, 1992; Dalecky et al., 2005; Schrempf et al., 2011). The lower queen egg-laying rates in polygynous societies could be the result of direct aggressive competition between queens as observed in *Cardiocondyla* (Yamauchi et al., 2007) and *Odontomachus* (Medeiros et al., 1992) ant queens or reciprocal pheromone inhibition as indicated in the fire ant *S. invicta* (Vargo, 1992). Moreover, Fletcher et al. (1980) hypothesized that queens could indirectly compete for a limited workforce that is for food, grooming or care of their larvae, albeit they later rejected this idea (Fletcher and Blum, 1983). During several months of experiments, we did not observe a single aggressive act between polygynous queens. We can therefore reject the first hypothesis. If reciprocal pheromone inhibition plays a role, we would expect the reciprocal negative effect on queen reproduction to decrease when the physical distance between them increases (Boulay et al., 2007). Instead, we found the opposite to be true. Queens residing closer to each other showed higher egg production, making this explanation for the lower queen fecundity in polygynous colonies unlikely. This leaves the hypothesis of competition for the workforce. We found that queens in polygynous colonies were groomed or fed as often by workers as those in monogynous colonies. However, egg production was positively correlated to colony size, suggesting that worker number per queen might indeed be a key determinant of reproductive output in *T. rugatulus* queens. Strikingly, microgynes laid as many eggs as the

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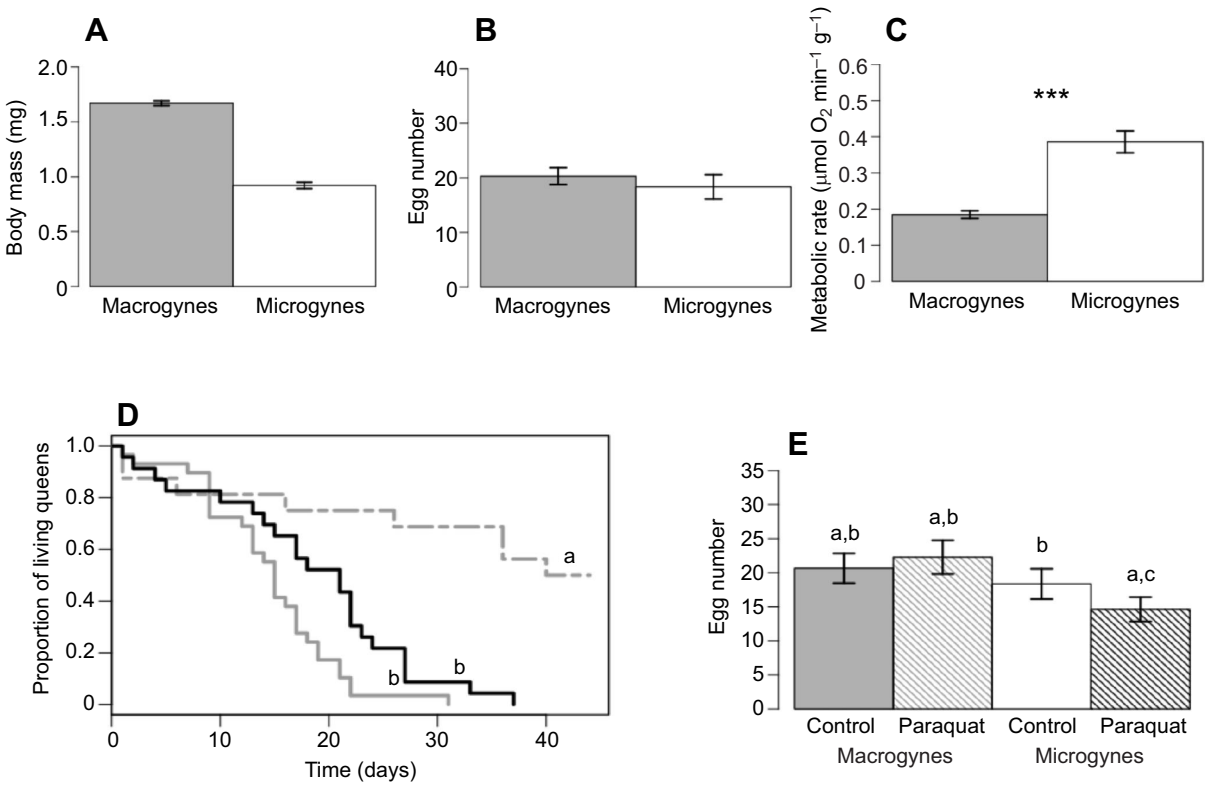

Fig. 3. Comparison of macrogyne and microgyne queens. (A) Body mass. (B) Egg number. (C) Metabolic rate before paraquat exposure. (D) Queen survival (dashed grey line, control; solid grey line, paraquat-macrogyne; solid black line, paraquat-microgyne). (E) Egg production under paraquat administration. Letters indicate results from pairwise *post hoc* comparisons with Bonferroni correction. ***P<0.001.

much larger macrogynous queens. We cannot rule out that this is a result of the small size of our experimental colonies, in that limited worker care might have masked the effect of queen morph on egg production (Rüppell et al., 1998). In the field, colony size is on average about 175 workers – more than 3 times the number of our experimental colonies – but very large colonies can contain up to 1900 workers in *T. rugatulus* (M.A.N., unpublished data). In these large societies, queens might be closer to their reproductive limits, so that more queens in a colony might increase the overall egg production (Negroni et al., 2016; Rüppell et al., 1998).

Many ants show variation in queen number between colonies, but in only a few species, such as *T. rugatulus*, is this variation in social organization linked to two distinct queen morphs, which have been shaped by selection. To shed light on proximate mechanisms underlying these divergent queen phenotypes, we contrasted the metabolic rate, survival under oxidative stress and fat body transcriptomes of microgynes and macrogynes. We demonstrate that larger queens have a lower metabolic rate than smaller ones. Although social insect queens are among the most fertile insects, we found in *T. rugatulus* that the scaling between body mass and metabolic rate in queens follows a typical trend observed in other insects (see Fig. S2c; Chown et al., 2007). Thus, neither body size nor metabolic rate was linked to reproduction, indicating that selection for fecundity in social insect queens did not affect the relationship between body mass and metabolic activity.

We detected no difference in survival under paraquat-induced oxidative stress between the two queen morphs. While the link between paraquat-induced stress resistance and longevity is well established in *D. melanogaster* (Arking et al., 1991; Harshman et al., 1999; Lin et al., 1998; Vermeulen et al., 2005), data are scarce from social insects (Lucas and Keller, 2014; Lucas et al., 2017). Despite the fact that oxidative stress experiments with paraquat have to be treated with care because of possible confounding factors (Meitern et al., 2013), we used this method to gain first insight into potential caste-specific differences in lifespan, assuming that individuals with a higher life expectancy would survive better under oxidative stress (Rzezniczak et al., 2011). Mortality under normal laboratory conditions is so low, so we would have been unable to detect potential variation in our study, given that *Temnothorax* queens can live up to 20 years (Plateaux, 1986). Our results suggest that the two queen morphs do not differ in their oxidative stress resistance, potentially indicating no variation in lifespan as well. Despite their stark differences in body size and reproductive behaviour, microgynes and macrogynes thus exhibited a similar fecundity and survival rate under oxidative stress. Even in mixed colonies, the reproductive contribution did not differ between microgyne and macrogyne queens, pointing to body size per se being of lower importance for queen fecundity in this social insect. Only under oxidative stress did we detect fecundity differences, in that microgynes were unable to maintain a high egg-laying rate, potentially indicating a trade-off between egg production and somatic maintenance (Kirkwood, 1977). From an evolutionary point of view, macrogynes should be under stronger selection to maintain high egg-laying rates, even under stress, as they reside in monogynous colonies and are thus often the sole reproductive queen, whereas microgynes commonly share reproduction with other queens (Negroni et al., 2016).

Our transcriptome analyses revealed clear differences in gene expression between the two morphs. Macrogynes and microgynes differed in the expression of candidate genes involved in the insulin growth-factor signalling pathway, which is well known as a major regulator of lifespan and reproduction in solitary species (Flatt et al., 2013). This stands in contrast to our phenotypic measurements that

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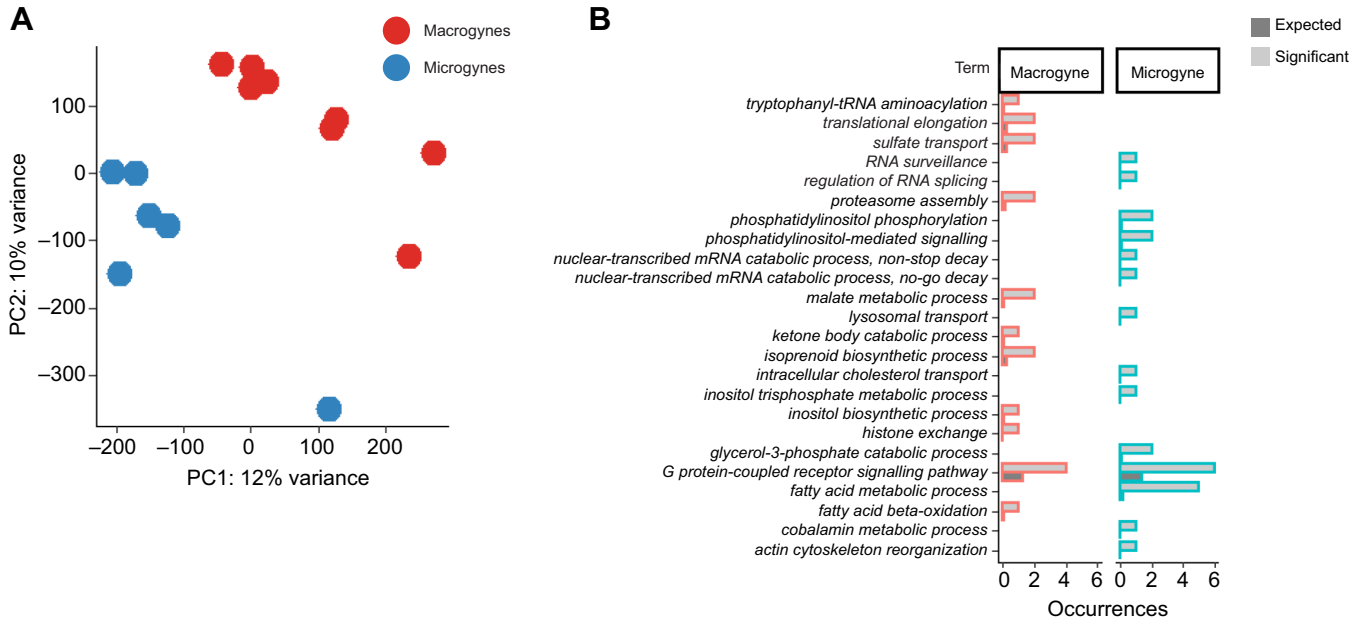

Fig. 4. Gene expression comparison between queen morphs. (A) Principal component analysis based on RNA-Seq data of all samples coloured by queen morph (red, macrogyne; blue, microgyne). (B) Functional enrichment with all significantly enriched functions represented using bar graphs, showing the actual number of terms in the set of candidate genes as well as the expected number of terms.

revealed no difference in fecundity and probably longevity between the two queen morphs. However, the enrichment analyses showed that macrogynes and microgynes vary in the activation of various metabolic pathways, which is consistent with our phenotypic findings, in particular with the metabolic rate measurements. The most significant among them is malate metabolism, which was suggested to prevent senescence and cancerous cell proliferation (Wiley and Campisi, 2016). Although the directionality of the molecular physiological shifts remains unresolved, the differential activation of the malate pathway could potentially contribute to the absence of differences in paraquat resistance between the morphs, despite a higher metabolic rate in microgynes. Finally, our transcriptome analysis revealed that macrogynes might degrade more damaged proteins as they upregulate genes with a proteasome production functionality. Proteasome activity has been reported to have a positive effect on lifespan (Chondrogianni and Gonos, 2008). The gene expression and functional enrichment analyses may therefore indicate that the queen morphs differ in their physiology, reflecting their adaptation to their life-history strategies.

Conclusions

Our experimental approach allowed us to disentangle the influence of various factors important in the evolution of life-history traits in relation to social life. Contrary to findings in most solitary species (Stearns, 1992), body size had no effect on female fecundity in our ant model. Instead, the major determinant of queen and colony egg production was the number of workers present in a colony. This is in line with the superorganism concept, where colony size replaces body size as an important colony-level life-history trait (Shik et al., 2012; Negroni et al., 2016). Increasing numbers of reproductives in a colony led to a reduced egg-laying rate per queen despite a moderate reproductive skew (Vargo and Fletcher, 1989; Vargo, 1992). How can the smaller microgynes keep up with the larger macrogynes in egg production? Our data indicate that smaller queens receive more food from workers and have a higher metabolism, which was also corroborated by our transcriptome analyses. Surprisingly, the higher metabolism did not increase their susceptibility to paraquat-induced oxidative stress, pointing to a less direct relationship between metabolic activity and lifespan than commonly presumed (Speakman et al., 2004; Brand, 2000). Our transcriptome analysis revealed that queen morph-specific differences in gene expression are associated with longevity pathways such as malate metabolism, proteasome activity or insulin growth-factor signalling. While the two morphs showed similar egg-laying rates and survival under oxidative stress, they differed in their metabolism as well as in their gene expression, which may indicate two alternative physiological routes to a similar fecundity and longevity phenotype. Our study adds further insight into life-history relationships and highlights the unique characteristics of insect societies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.F.; Methodology: M.A.N., M.O., A.-S.R., S.F.; Software: M.S., B.F.; Validation: M.S.; Formal analysis: M.A.N., M.S., M.O., A.-S.R., B.F.; Investigation: M.A.N., M.O., A.-S.R., B.F.; Resources: M.A.N., S.F.; Data curation: M.A.N., M.S., S.F.; Writing - original draft: M.A.N.; Writing - review & editing: M.S., M.O., A.-S.R., B.F., S.F.; Visualization: M.A.N., M.S., B.F., S.F.; Supervision: M.A.N., B.F., S.F.; Project administration: S.F.; Funding acquisition: B.F., S.F.

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Data availability

All sequence data for this study are archived at NCBI's Short Read Archive (SRA) with links to BioProject accession number PRJNA549424, SRR9652941–

SRR9652954 (https://www.ncbi.nlm.nih.gov/bioproject/). The codes as well as the input and raw data are available from Google Drive: https://drive.google.com/drive/folders/1-lJVUCP0cbsM3knLPmKXjUEZmRdkfD4w?usp=sharing

Supplementary information

Supplementary information available online at
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